ORAL ADMINISTRATION OF HYDROGEN WATER PREVENTS CHRONIC ALLOGRAFT NEPHROPATHY IN RAT RENAL TRANSPLANTATION

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Abbreviations: reactive oxygen species (ROS), chronic allograft nephropathy (CAN), hydrogen water (HW), regular water (RW), mitogen activated protein kinase (MAPK), c-Jun NH 2-terminal kinase (JNK), extracellular related kinase (ERK), blood urea nitrogen (BUN), creatinine (Cr), creatinine clearance (CCr)

ABSTRACT: (210 words)

Tissue injury, induced by reactive oxygen species (ROS), contributes to the development of chronic allograft nephropathy (CAN; also known as interstitial fibrosis and tubular atrophy with unknown etiology [IF/TA]) after renal transplantation. Molecular hydrogen gas can act as a ROS scavenger. We hypothesized that administration of hydrogen water (HW) would ameliorate CAN by scavenging ROS. Using a rat model of kidney transplantation, LEW rat allografts were orthotopically transplanted into BN rat recipients that had undergone bilateral nephrectomy. Molecular hydrogen was dissolved in water (>0.8 mM) and recipients were given either regular water (RW) or HW from day 0 to day 150, or death. RW-treated animals experienced a gradual decline in creatinine clearance, associated with proteinuria, which ultimately led to graft failure secondary to CAN. In contrast, HW administration improved allograft function, slowed the progression of CAN, and improved overall survival. HW also reduced oxidant injury and inflammatory mediator production. Additionally, inflammatory signaling pathways (mitogen activated protein kinases) were less activated in renal allografts from HW-treated rats compared to the RW-treated group. These data indicate that orally administered HW is an effective antioxidant and anti-inflammatory agent that can prevent CAN and improve survival in a model of rodent renal transplantation. HW may be of therapeutic value in the setting of transplantation.

INTRODUCTION:

Chronic kidney disease is the 9th leading cause of death in the United States accounting for over 40,000 deaths annually.[1] Despite advances in renal replacement therapy, transplantation remains the preferred treatment for suitable candidates.[2] However, in spite of improved postoperative immunosuppression regimens, the 10-year graft survival rates are 55% and 75% for cadaveric and live donor kidney allografts, respectively. The vast majority of late failures are attributable to chronic allograft nephropathy (CAN), recently reclassified as interstitial fibrosis and tubular atrophy with unknown etiology (IF/TA).[3-6] The clinical course of CAN is characterized by a progressive deterioration in renal function manifested by increasing renal hypertension and proteinuria. Presently, no specific treatment is available for chronic rejection in clinical transplantation, despite a number of successful approaches in animal models, including the use of macrophage inhibitors, angiotensin converting enzyme (ACE) inhibitors and endothelin A receptor antagonists. [7-10]

A number of factors contribute to the development of CAN, including immunologic (e.g. acute rejection) and non-immunologic (e.g. ischemia reperfusion injury) factors.[11, 12] Oxidative stress is believed to be a common pathway that leads to both immunologic and non-immunologic stress in the setting of kidney transplantation and, ultimately, the development of CAN.[13] Markers of oxidative stress, such as plasma lipid peroxidases, are increased while antioxidant markers, including glutathione (GSH), superoxide dismutase (SOD) and glutathione peroxidase, are decreased in the setting of CAN.[14-17] Despite this association, few studies have attempted to examine the effect of antioxidants on kidney allograft outcomes and those that have yielded mixed results. Vitamin E (alpha-tocopherol) supplementation did not prevent allograft injury in a model of CAN[16]; however, in the same model, L-arginine did attenuate proteinuria and glomerulosclerosis.[18] Furthermore, a clinical trial using recombinant human SOD resulted in significantly decreased acute and chronic rejection.[19] Therefore, based on the seemingly

conflicting results of these few studies, there is a need for additional investigations into the applicability of antioxidants for the prevention of CAN.

Molecular hydrogen has recently been shown to have therapeutic value as an antioxidant through its ability to selectively reduce cytotoxic reactive oxygen species (ROS).[20] Inhaled hydrogen gas (~ 4% H_2 in air) can reduce infarct size in rat models of focal cerebral and myocardial ischemia reperfusion injury.[20, 21] More recently, our group reported that perioperative hydrogen inhalation (2%) significantly ameliorates intestinal transplant injury and prevents remote organ inflammation via its antioxidant effects.[22] Drinking water containing a therapeutic dose of hydrogen (hydrogen water; HW) represents an alternative mode of delivery of molecular hydrogen. The primary advantages of HW are that it is a portable, easily administered and safe means of delivering of molecular hydrogen. Therefore, it may be of potential therapeutic value in the treatment of oxidative stress-induced pathologies. Interestingly, drinking HW, as well as inhaling hydrogen gas, can alleviate cisplatin-induced nephrotoxicity, which is known to be mediated, in part, by the accumulation of ROS that occurs secondary to the ability of cisplatin to inhibit the reducing form of glutathione[23] Consumption of HW *ad libitum* prevents the development of atherosclerosis in apolipoprotein E knockout mice, in part, through its ability to limit the amount and deleterious effects of oxidative stress in the blood vessels of these mice.[24] Furthermore, a clinical trial in type 2 diabetic patients given supplemental hydrogen water led to improved lipid and glucose metabolism compared to controls.[25] The aim of the present study was to determine the efficacy of HW in preventing CAN after allogeneic kidney transplantation in rats.

RESULTS:

Oral administration of hydrogen water increased local and systemic levels of hydrogen

To determine whether oral administration of HW results in increased local or systemic levels of molecular hydrogen, unoperated, naïve LEW rats were given HW to drink and, subsequently, the concentration of molecular hydrogen was measured in the kidney, as well as in the serum. Both

local and systemic concentrations of molecular hydrogen peaked approximately 15 minutes following ingestion (Figure 1A), proving that HW is an effective mode of delivery of molecular hydrogen. We also tested whether transplant recipients given long-term, daily HW had increased concentrations of circulating hydrogen. Similar kinetic changes in hydrogen concentration were observed in both LEW recipients with LEW grafts (control rats; syngeneic transplantation prevents CAN) and BN recipients with LEW grafts treated with HW for 60 days (Figure 1B). The baseline levels of the hydrogen detected in the circulation after 60 days of HW treatment was comparable to that of naïve animals, suggesting there was no hydrogen accumulation during long-term HW administration.

Oral administration of hydrogen water improves kidney function following allotransplantation.

We then sought to determine the effect of HW administration on kidney function following allotransplantation. Isograft recipients (LEW donor and LEW recipient) were included as controls, as these rats do not develop CAN. Using a model of kidney transplantation followed by bilateral nephrectomy, we found that rats receiving daily HW *ad libitum* had improved allograft function as measured by blood urea nitrogen (BUN), creatinine clearance (CCr), and proteinuria compared to those receiving regular water (RW) at 60 days posttranplantation (Figure 1C). These results suggest that HW can improve kidney function following allotransplantation.

Oral administration of hydrogen water improves weight gain and overall survival following allotransplantation.

To determine whether the improved allograft function that was observed in HW-treated recipients correlated with global parameters of well-being, we measured the weights of transplant recipients and found that the majority of animals that had undergone allotransplantation followed by administration of RW began to lose body weight approximately 40 days after transplantation.

This process was significantly diminished in the allograft recipients that received HW (Figure 2A). Furthermore, we found that weight loss proved to be a harbinger of allograft failure and, ultimately, death. HW-treated recipients exhibited a significant increase in survival (median survival >150 days) compared to RW-treated controls (median survival 78 days) (Figure 2B). These results show that the improved allograft function that was observed at 60 days posttranplantation in HW-treated recipients, as compared to RW-treated controls, also leads to improved survival outcomes.

Oral administration of hydrogen water prevents the progression chronic allograft nephropathy.

To determine if the improved allograft function and overall survival observed in HW-treated animals were attributable to decreased chronic rejection, histologic analysis was performed on allografts obtained 60 days posttranplantion from both RW- and HW-treated recipients. H&E staining of the allografts obtained from HW-treated recipients exhibited decreased evidence of the hallmarks of CAN, including less glomerulosclerosis and inflammatory cell infiltration, as compared to H&E staining of allografts obtained from RW-treated recipients (Figure 3A). Furthermore, Masson's trichrome staining and α-smooth muscle actin (αSMA) staining on allografts obtained from HW-treated recipients demonstrated less interstitial fibrosis and smooth muscle proliferation, respectively, compared to allografts obtained from RW-treated controls (Figure 3B, C). The expression of αSMA, indicating myofibroblast accumulation in the grafts, was mostly seen in the interstitial areas. There was no definitive, αSMA-positive staining in tubular epithelial cells. Histopathology of the control isografts from the same time point was similar to that of normal, naïve animals and was not affected by HW administration (data not shown). Additionally, immunohistochemistry for both CD3 and CD68 revealed fewer graft-infiltrating T cells and macrophages, respectively, in allografts obtained from HW-treated

recipients compared to those obtained from RW-treated controls (Figure 4A, B). These results proved to be statistically significant when the number of positive-staining cells per high power field was counted for each sample (Figure 4C). Taken in total, these histological results suggest that allografts from HW-treated recipients experienced less CAN than those from RW-treated controls.

Oral administration of hydrogen water is an effective antioxidant strategy in the setting of kidney allotransplantation.

As mentioned previously, molecular hydrogen possesses potent antioxidant properties. Furthermore, oxidant stress-induced tissue damage is believed to be a common pathway in many of the pathophysiologic mechanisms involved in the development of CAN. Therefore, due to the fact that HW administration resulted in increased local and systemic concentrations of molecular hydrogen, as well as decreased histologic evidence of CAN in kidney allograft recipients compared to RW-treated controls, we next determined if the protection from CAN seen with HW administration was accompanied by a decrease in markers of oxidative tissue injury. Tissue malondialdehide (MDA) levels, which indicate lipid peroxidation in cells and tissues, were significantly decreased in allografts obtained 60 days posttranplantion from HW-treated recipients compared to those obtained from RW-treated controls (Figure 5A). Furthermore, immunohistochemistry performed on allografts obtained from HW-treated recipients exhibited less 4-Hydroxy-2-nonenal (HNE) and peroxynitrite staining compared to that seen in allografts obtained from RW-treated controls (Figure 5 B,C). These results demonstrate that the local and systemic levels of molecular hydrogen that are achieved via the administration of HW are sufficient to effectively reduce oxidative stress-induced tissue damage in the setting of kidney allotransplantation.

Oral administration of hydrogen water decreases the local production of inflammatory markers in the setting of kidney allotransplantation.

One mechanism by which oxidative stress leads to the development of chronic rejection is by increasing the production of inflammatory cytokines.[26, 27] Therefore, because HW administration led to decreased oxidative stress and slowed the progression toward CAN in kidney allografts, we next determined whether HW treatment was also associated with a decrease in local inflammatory cytokine production. qRT-PCR revealed significantly lower levels of interleukin (IL)-6, tumor necrosis factor-alpha (TNFα), intracellular adhesion molecule-1 (ICAM-1) and interferon-gamma (IFNγ) mRNA in kidney allografts obtained from HW-treated recipients 60 days posttranplantion as compared to those obtained from RW-treated controls (Figure 6A-D). These results indicate that HW administration can attenuate the local production of inflammatory markers in the setting of kidney allotransplantion.

Oral administration of hydrogen water decreases the activation of inflammatory signaling cascades following kidney allotransplantation.

Inflammatory intracellular signaling pathway activation (most notably activation of mitogen-activated protein kinases; MAP kinases) is a well-described event that contributes to the progression of kidney allografts toward chronic rejection.[28-30] Oxidative stress can activate MAP kinase signaling, which ultimately contributes to the proliferation of mesangial cells in the setting of diabetic nephropathy.[31] Mesangial cell proliferation is also involved in the development of CAN. Therefore, we next determined whether HW administration could suppress MAP kinase activation in kidney allografts. Western blot analysis demonstrated that MAP kinases, including c-Jun N-terminal kinase (JNK), p-38, extracellular signal-regulated protein kinase (ERK1/2) as well as upstream kinase cascades (MEK-1), were less activated in allografts obtained from HW-treated recipients than in allografts obtained from RW-treated recipients (Figure 7). These results

indicate that HW administration can inhibit intracellular signaling pathways that are known to contribute to the development of CAN in the setting of kidney transplantation.

DISCUSSION:

In this study, we found that both allograft function and overall survival were improved in rats that had been fed a diet supplemented with HW. Allografts from HW-treated rats exhibited less infiltration of inflammatory cells and suppressed activation of intragraft inflammatory signaling pathways. The allografts from the HW-treated rats manifested fewer markers of oxidative stress and, ultimately, fewer progressed toward CAN as compared to controls. These results indicate that HW represents a potentially novel therapeutic strategy in the prevention of CAN in kidney transplantation.

Molecular hydrogen is produced continuously under normal physiologic conditions, primarily during the fermentation of non-digestible carbohydrates by intestinal bacteria in the large intestine. This physiological production of hydrogen gas may be responsible for the baseline levels of hydrogen detected in circulation. It is excreted as flatus, further metabolized by gut flora, or exhaled as a natural component of abdominal gas. However, molecular hydrogen has known physiologic roles during conditions of homeostasis. Recent evidence indicates that inhaled hydrogen gas has antioxidant and anti-apoptotic properties that can protect organs from ischemia-reperfusion-induced injury by selectively scavenging detrimental ROS. The mechanism of action of inhaled hydrogen gas in these models involves its ability to prevent oxidative damage, as indicated by decreased nucleic acid oxidation and lipid peroxidation.[20, 21] Although the concentration of gaseous molecular hydrogen used in the above studies (~ 4%) is lower than the threshold at which it is known to be flammable (4.6%), flammability is still a realistic concern which may limit the translational applicability of inhaled molecular hydrogen. Therefore, HW represents a novel and easily translatable method of delivery of molecular hydrogen. To our knowledge, this is the first report describing the preventative effects of molecular hydrogen

delivered in water containing therapeutic doses on the development of chronic rejection in the setting of kidney allotransplantation and, as such, represents a potentially novel and easily applicable solution to a difficult clinical scenario (i.e. CAN). The major novel findings of the present study are that i) hydrogen water improves allograft function and overall survival by preventing CAN in a rodent model of kidney transplantation, doing so in part by ii) reducing oxidative stress-induced damage and iii) reducing the activation of inflammatory signaling pathways and cytokine production.

The basis for the present study was the fact that oxidative stress is believed to be a common pathway that leads to the development of chronic rejection in kidney transplantation. As mentioned, markers of oxidative stress are elevated in kidney transplant recipients and, in contrast, markers of antioxidant pathways are diminished. Mechanistically, ROS activate inflammatory intracellular signaling pathways in vascular smooth muscle cells,[28] induce the epithelial-to-mesenchymal transition,[32, 33] participate in extracellular matrix deposition by mesangial cells[34], and contribute to renal tubular atrophy through apoptosis[35, 36] and inflammation[26, 27] - all of which are key processes involved in the pathogenesis of CAN.

Given the association described above between oxidative stress and the development of CAN, the finding that HW administration effectively decreases the intragraft accumulation of markers of oxidative stress, such as MDA, 4-HNE and peroxynitrite, suggests that the antioxidant properties of molecular hydrogen are likely responsible for the beneficial effects on allograft function and the prevention of progression of CAN that were observed in this rat model of kidney allotransplantation. We examined the intragraft expression of TNFα, IL-6, IFNγ and ICAM-1 at the mRNA level, as surrogate markers of the deleterious processes downstream from oxidative damage, and found less expression in HW-treated recipients of these inflammatory cytokines, which are well-described mediators of the fibrogenesis phase of CAN.[37] Furthermore, MAP kinase signaling, which is known to be induced by oxidative damage in the setting of kidney transplantation, was decreased in HW-treated recipients. MAP kinase signaling contributes to the

development of CAN by mediating the action of growth factors, such as TGFβ,[38] participating in the proliferation of vascular smooth muscle cells[28] and the extracellular matrix[29], and contributing to the intragraft infiltration of mononuclear inflammatory cells through the production of chemoattractants.[30]

Therefore, we conclude that HW, through its ability to act as an effective method of delivery for molecular hydrogen, can reduce the development of CAN in a rat model of kidney allotransplantation. This is improves allograft function and overall survival in HW-treated recipients. The mechanism of protection afforded to HW-treated recipients likely involves the ability of molecular hydrogen to reduce oxidative stress-induced damage, which is believed to be an upstream mediator that contributes to the ultimate development of CAN. Consequently, HW may be an effective and novel tool in the clinical armamentarium against oxidative stress-induced pathologies, in general, and CAN, in particular.

MATERIALS AND METHODS:

Animals

Inbred male Lewis (LEW, $RT1^{l}$) and Brown Norway (BN, $RT1^{n}$) rats weighing 200-250 g were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Animals were maintained in cages in a specific pathogen-free facility at the University of Pittsburgh and fed a standard diet with free access to water. All procedures were performed according to the guidelines of the Institutional Animal Care and Use Committee at the University of Pittsburgh and the National Research Council's Guide for the Humane Care and Use of Laboratory Animals.

Kidney transplantation

Orthotopic kidney transplantation was performed using a previously described technique.[39, 40] In short, after intravenous heparinization (300 U), the donor's left kidney was removed with the left renal artery in continuity with a short aortic segment and the left renal vein with a patch of vena cava. The excised graft was flushed with 3 ml of UW solution (Viaspan, Du Pont, Wilmington,

DE). The left kidney graft was orthotopically transplanted into the recipient by end-to-side microvascular anastomoses between graft aorta and recipient infrarenal abdominal aorta, and between graft renal vein and recipient infrarenal vena cava with 10-0 Micrin suture. Both native kidneys of the recipient were removed, and end-to-end ureteral anastomosis was performed using 10-0 Micrin suture. Recipients received prophylactic antibiotics (Cefotetan disodium, 100 mg/kg, intramuscular injection) for 3 days following the transplantation.

Oral administration of hydrogen water (HW)

HW was produced by Blue Mercury Inc. (Tokyo, Japan) using a HW-producing apparatus, by which molecular hydrogen gas was dissolved in water under a pressure of 0.4 MPa, as previously described.[23, 24, 41] The HW (500 mL, hydrogen concentration >0.6 mM) was stored in an aluminum bag and placed into a glass vessel twice a day. Control animals were treated with RW, generated by degassing HW via gentle stirring for 24 hours.

Determination of hydrogen levels in blood and tissue

HW (3 mL) was orally administered by gavage to naïve LEW rats or transplant recipients that had already been treated with HW > 60 days. Arterial blood and kidney tissue were taken at 15, 30 and 60 minutes after oral administration of HW. Blood or homogenized kidney tissue was placed in a glass tube and air-phase hydrogen levels were measured by gas-chromatography (Biogas analyzer BAS-1000, Mitleben, Osaka, Japan). [22]

Experimental groups

Four experimental groups were analyzed: LEW to LEW syngeneic grafts treated with RW (Group 1), LEW to LEW syngeneic grafts treated with HW (Group 2), LEW to BN allogeneic grafts treated with RW (Group 3) and LEW to BN allogeneic grafts treated with HW (Group 4). Recipients of allografts received daily intramuscular injections of tacrolimus (FK506, Astellas Pharmaceutical, Tokyo, Japan) at a dose of 0.5 mg/kg for 7 days (days 0–6) while those of isografts received no immunosuppressant. Recipients were sacrificed at 60 or 150 days after transplantation, and blood and kidney graft samples were obtained after being cleared of blood by

flushing with Lactated Ringers. Kidney tissue was snap-frozen and stored at -80°C until use or fixed in 10% buffered formalin for routine histopathology. For survival study experiments, recipient animals were followed until recipient death due to graft failure or for 150 days after transplantation.

Evaluation of graft function

Renal graft function was assessed by measuring plasma creatinine (P[Cr]) and BUN levels, as well as urinary protein excretion and urinary creatinine (U[Cr]) levels using an autoanalyzer (Beckman Instruments, Fullerton, CA). Creatinine clearance (CCr; mL/min) was calculated using the formula (CCr; ml/min) = (U[Cr] x urine volume)/(P[Cr] x time). Urine samples were collected using metabolic cage systems.

Histopathological analysis

Formalin-fixed graft tissues were paraffin embedded, cut into 5 μm sections, and stained with hematoxylin/eosin, modified Masson's trichrome, or Verhoeff's elastic tissue stain (Rowley Biochemical Institute, Danvers, MA). Sections were also immunohistochemically analyzed using the avidin-biotin-peroxidase complex method after antigen retrieval and incubation with mouse anti- SMA (α-SMA, DAKO, Carpinteria, CA), monoclonal anti-rat CD68 (ED1, Serotec, Raleigh, NC) or monoclonal anti-rat CD3 (Serotec), followed by incubation with LSAB®+ horseradish peroxidase (DAKO). Tissue oxidative injury was also assessed by immunostaining with rabbit polyclonal anti-nitrotyrosine (Santa-Cruz Biotechnology, Santa Cruz, CA) and mouse monoclonal anti-4HNE antibody (JAICA, Shizuoka, Japan), followed by incubation with biotinylated anti-mouse IgG antibody (Vector Laboratories) and avidin-biotin-peroxidase complex (Vector Laboratories). Diaminobenzidine was used as the peroxidase substrate. In each study, a set of sections was stained without the primary antibody as a negative control.

Assessment of tissue MDA

Tissue MDA levels, were assessed using the BIOXYTECH® MDA-586™ kit (OxisResearch, Portland, OR) as previously described.[42, 43] Kidney samples were homogenized in buffer (pH 7.9)

containing 5 mM butylated hydroxytoluene to prevent sample oxidation and stored at -80°C. Once all samples were collected, they were thawed on ice and 10 µg of probucol was added to further minimize oxidative reactions, then *N*-methyl-2-phenylindole (NMPI) in 25% methanol, 75% acetonitrile was added to the supernatants, followed by addition of 1x HCI and incubation at 45 °C for 60 min. A standard curve using 1,1,3,3-tetra-methoxypropane (TMOP), which generates free MDA during the acid hydrolysis step, was also prepared. All samples and standards were centrifuged (10,000 g, 10 minutes) and absorbance at 586 nm was measured using the Spectronic Biomate 3 (Thermo Scientific, MA) according to the manufacturer's protocol.

SYBR green real time RT-PCR

The levels of mRNAs for IL-6, ICAM-1, TNFα, IFNγ and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were quantified in duplicate using SYBR Green two-step, real-time RT-PCR, as previously described.[42, 43] Briefly, 1 µg of RNA from each sample was used for reverse transcription with oligo dT primers (Invitrogen, Carlsbad, CA) and Superscript II enzyme (Invitrogen) to generate first-strand cDNA. The PCR reaction mixture was prepared using SYBR Green PCR Master Mix (PE Applied Biosystems, Foster City, CA). Each sample was analyzed in duplicate using the conditions recommended by the manufacturer. Gene expression was normalized with GAPDH mRNA content.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blots

Cytoplasmic protein was isolated from the kidney grafts, and SDS-PAGE was performed using standard protocols. Total and phospho-protein levels were determined by western blot using primary rabbit polyclonal antibodies and secondary goat anti-rabbit antibodies (1:10,000, Pierce Chemical, Rockford, IL), as previously described.[44] The following primary antibodies were used: anti-phosphorylated (p)-ERK1/2 and anti-total (t)-ERK1/2 (Santa Cruz), anti-p-p38 MAP kinases,

anti-p-c-Jun N-terminal kinase (JNK), anti-t-p38, anti-t-JNK, anti-p-MEK, anti-t-MEK (all from Cell Signaling Technology, Beverly, MA).

Statistical analysis

Recipient survival was plotted using the Kaplan-Meier method, and the differences between groups were analyzed using the log-rank test. The other results were expressed as mean with standard deviation (SD). Statistical analysis was performed using analysis of variance (ANOVA) and the F-test with Bonferroni *post hoc* group comparisons, where appropriate. A probability level of $p<0.05$ was considered to be statistically significant with 95% confidence interval.

FIGURE LEGENDS:

Figure 1: Hydrogen water administration improves kidney allograft function.

(A) HW (3 mL) was orally administered to naïve LEW rats by gavage. Arterial blood and kidney tissue were taken at 15, 30 and 60 minutes after oral administration of HW. Hydrogen concentration in blood and homogenized kidney tissue increased within 15 minutes and then returned to the basal levels (n=3). (*$p<0.05$ vs. 0 minute) (B) Kinetic analysis of the blood hydrogen levels after HW (3 mL) administration in the transplanted recipients that had been given HW for 60 days were performed. Both isograft recipients (n=3) and allograft recipients (n=3) showed similar changes in circulating hydrogen concentrations. (C) Male LEW rats were used as donors for either syngeneic (LEW recipient) or allogeneic (BN recipient) renal transplantation. Recipients were given either regular water (RW) or hydrogen-rich water (HW) *ad libitum* after transplantation resulting in a total of 4 experimental groups: isograft given regular water (Iso/RW, n=8) or hydrogen water (Iso/HW, n=8); or allograft given regular water (Allo/RW, n=18) or hydrogen water (Allo/HW, n=17). The recipients receiving daily HW had improved allograft function as measured by blood urea nitrogen (BUN), creatinine clearance (CCr), and 24h urinary protein excretion compared to those receiving RW at 60 days posttranplantation. (*$p<0.05$ vs Allo/RW)

Figure 2: Hydrogen water administration improves long term survival following kidney allotransplantation.

(A) Although the recipients with isografts showed fair body weight gains regardless of intake of HW, the recipients with allografts given RW gradually lost body weight by 60-80 days after transplantation. The recipients given HW showed better body weight gain compared to those without HW. (B) In correlation with improved graft function, survival of allograft recipients was significantly prolonged with oral administration of HW. (Iso/RW; n=5, Iso/HW; n=5, Allo/RW; n=13, Allo/HW; n=12, Kaplan-Meyer, log-rank test, $p<0.05$ Allo/RW vs. Allo/HW)

Figure 3: Hydrogen water administration decreases chronic allograft nephropathy after kidney allotransplantation.

Hematoxylin/eosin (A) Masson's trichome (B) or α-smooth muscle actin (C) staining was performed 60d posttranplant to assess histologic and/or immunohistochemical evidence of chronic allograft nephropathy. Images are representative of 4 separate, individual grafts from each experimental group. Original magnificantion; x200.

Figure 4: Hydrogen water administration decreases intragraft inflammatory cell infiltration following kidney allotransplantation.

CD3-positive (A) and CD68-positive (B) infiltrating cells were assessed by immunohistochemistry and quantitated as number of positive-staining cells per HPF (400X) (C). Images are representative of 5 individual animals for each group. (n=5 for each group, original magnificantion; x200, * $p<0.05$ vs Allo/RW)

Figure 5: Hydrogen water administration decreases intragraft markers of oxidative stress following kidney allotransplantation.

(A) Intragraft malondialdehyde (MDA) levels were quantitated using a commercially available kit. MDA levels in the allografts treated with RW increased 60 days after transplantation. HW significantly reduced tissue MDA levels. (n=5, *$p<0.05$ vs Allo/RW). Additionally, immunohistochemistry for 4-Hydroxy-2-nonenal (HNE) (B) and peroxynitrite (C) was performed on formalin-fixed sections obtained from the allograft groups. Oxidative injuries were more prominent in the allografts with RW (Allo/RW) compared to those with HW (Allo/HW). Arrows indicate HNE-positive cells. (Original magnification; x400, Images are representative of 3 separate experiments).

Figure 6: Hydrogen water administration decreases the intragraft production of inflammatory cytokines after kidney allotransplantation.

IL-6 (a) TNFα (b) ICAM-1 (c) and IFNγ (d) mRNA levels in the kidney grafts taken 60 days after transplant were measured by real-time qRT-PCR analysis. Although allograft rejection caused upregulation of the mRNAs for these markers, HW significantly reduced the mRNA levels as compared with RW. (n=5, *$p<0.05$ vs Allo/RW)

Figure 7: Hydrogen water administration decreases intragraft inflammatory signaling cascade activation following kidney allotransplantation.

Western blot analysis demonstrated that MAP kinases (JNK, P-38, ERK1/2), as well as upstream kinase cascades (MEK-1), were less activated in allografts obtained from recipients that had HW-treated recipients compared to those obtained from RW-treated recipients. Images are representative of three independent experiments.

DISCLOSURE

None of the authors of this manuscript have a relationship with any company that may have a financial interest in the information contained in the manuscript.

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Figure 1

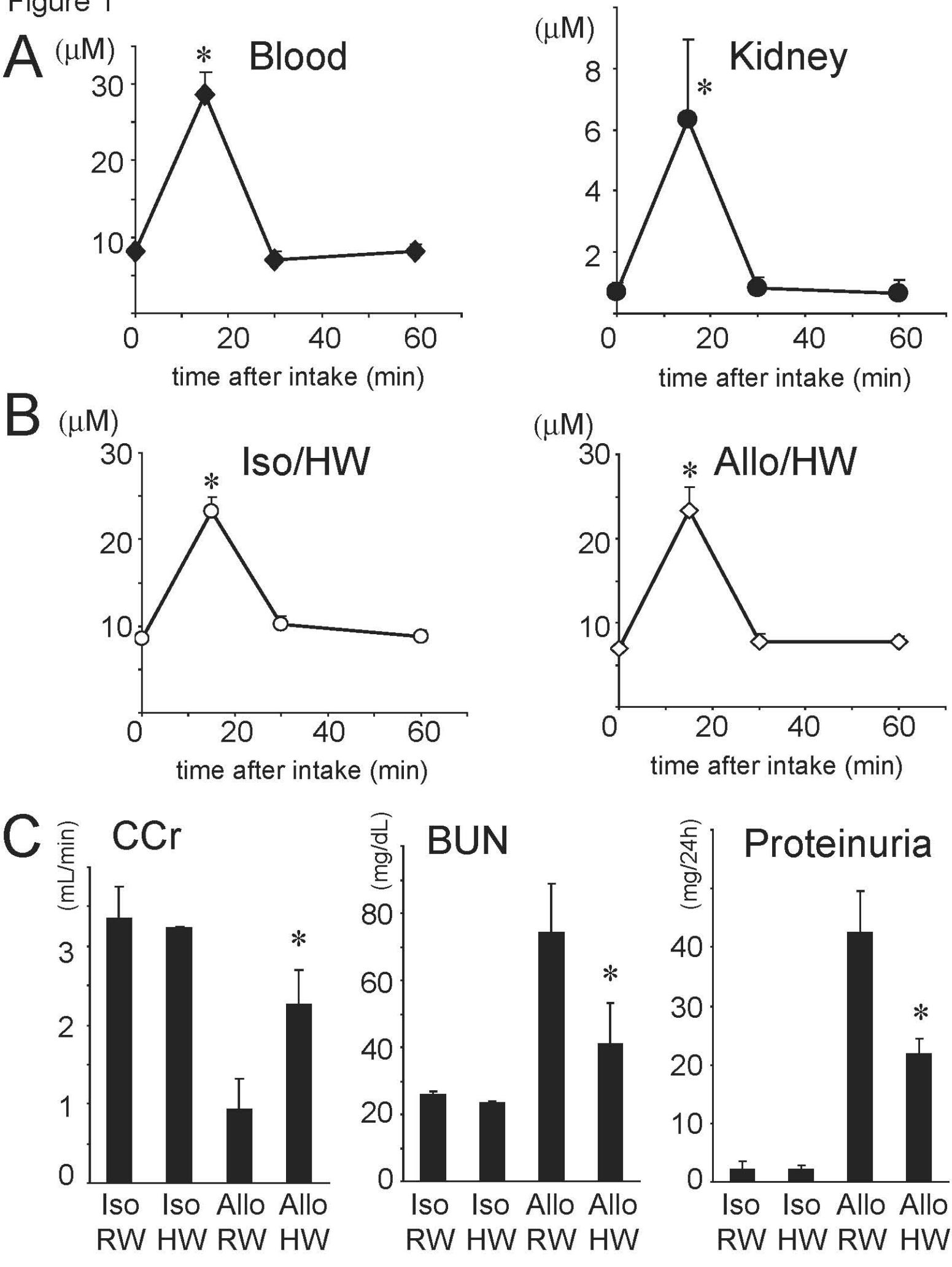
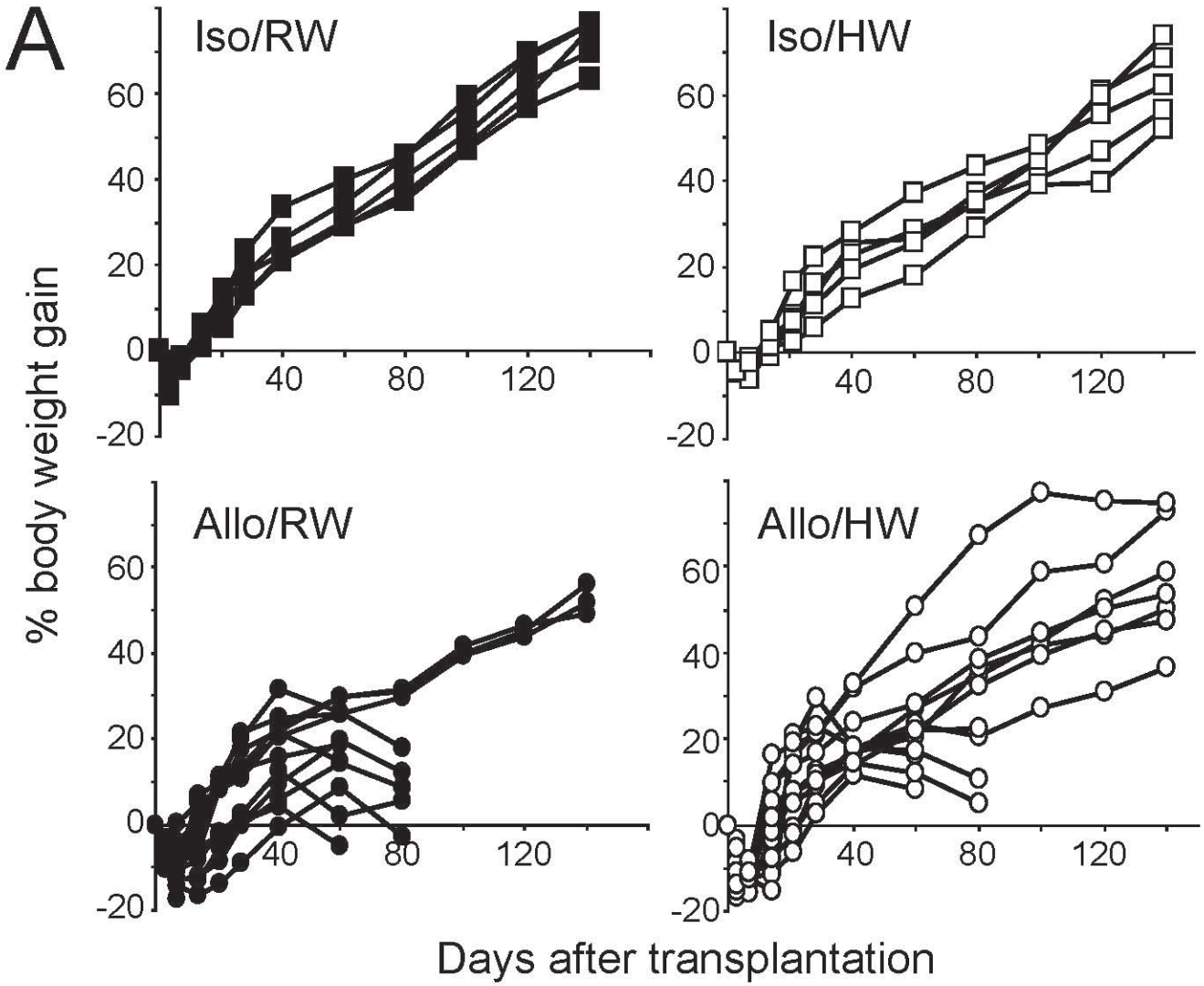

A
(μM)
Blood
30
20
10
*
0
20
40
60
time after intake (min)
(μM)
Kidney
8
6
4
2
*
0
20
40
60
time after intake (min)
B
(μM)
Iso/HW
30
20
10
*
0
20
40
60
time after intake (min)
(μM)
Allo/HW
30
20
10
*
0
20
40
60
time after intake (min)
C
CCr
(mL/min)
3
2
1
0
*
Iso RW
Iso HW
Allo RW
Allo HW
BUN
(mg/dL)
80
60
40
20
0
*
Iso RW
Iso HW
Allo RW
Allo HW
Proteinuria
(mg/24h)
40
30
20
10
0
*
Iso RW
Iso HW
Allo RW
Allo HW

Figure 2


A
Iso/RW
Iso/HW
Allo/RW
Allo/HW
% body weight gain
60
40
20
0
-20
40
80
120
Days after transplantation

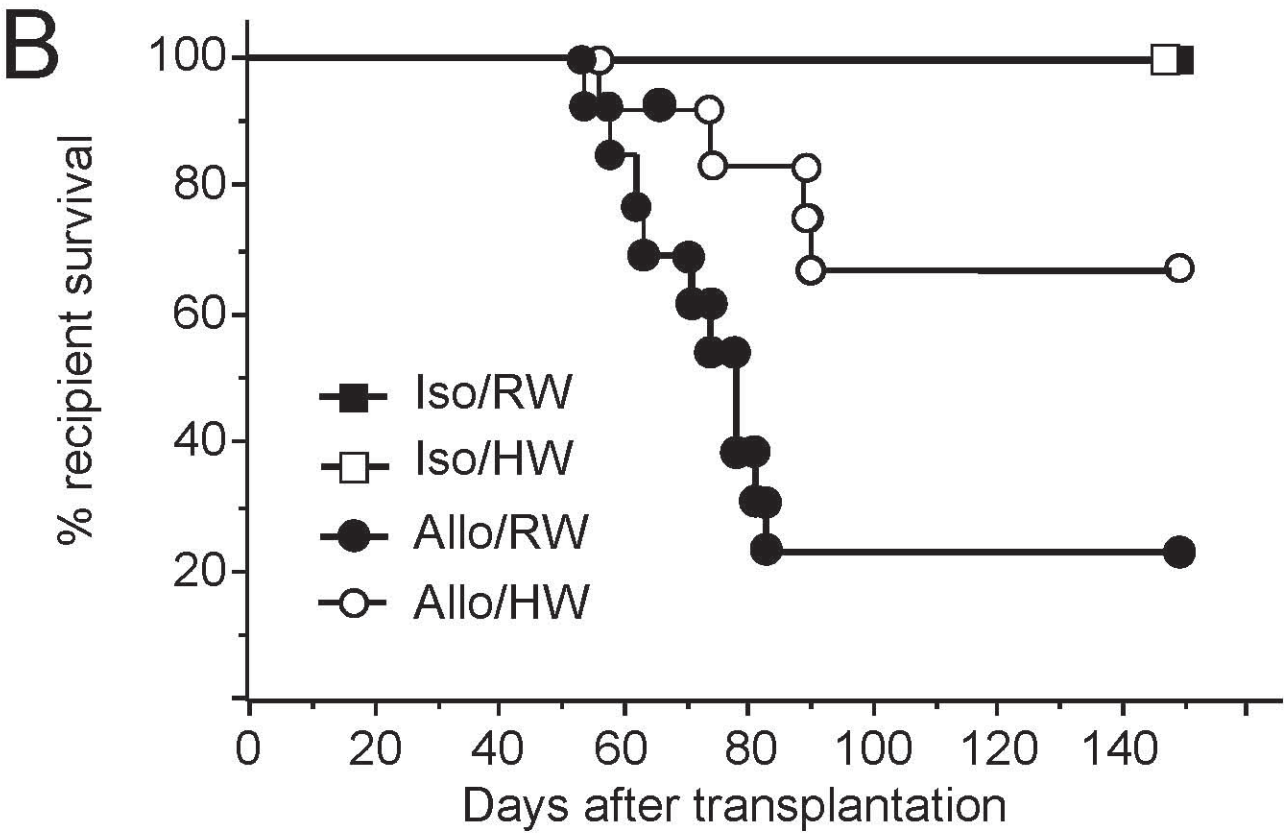
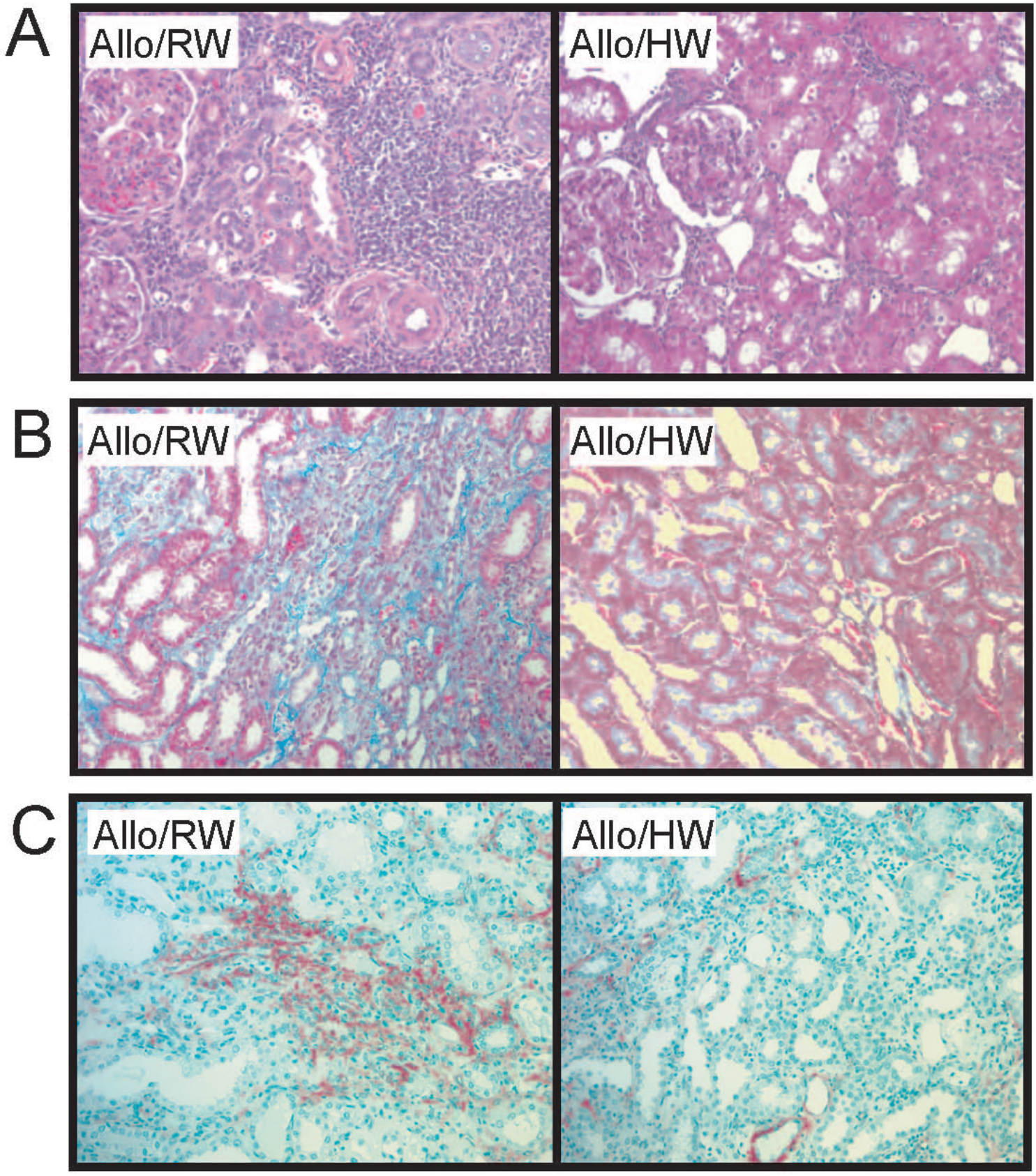

B
% recipient survival
100
80
60
40
20
Iso/RW
Iso/HW
Allo/RW
Allo/HW
0
20
40
60
80
100
120
140
Days after transplantation

Figure 3



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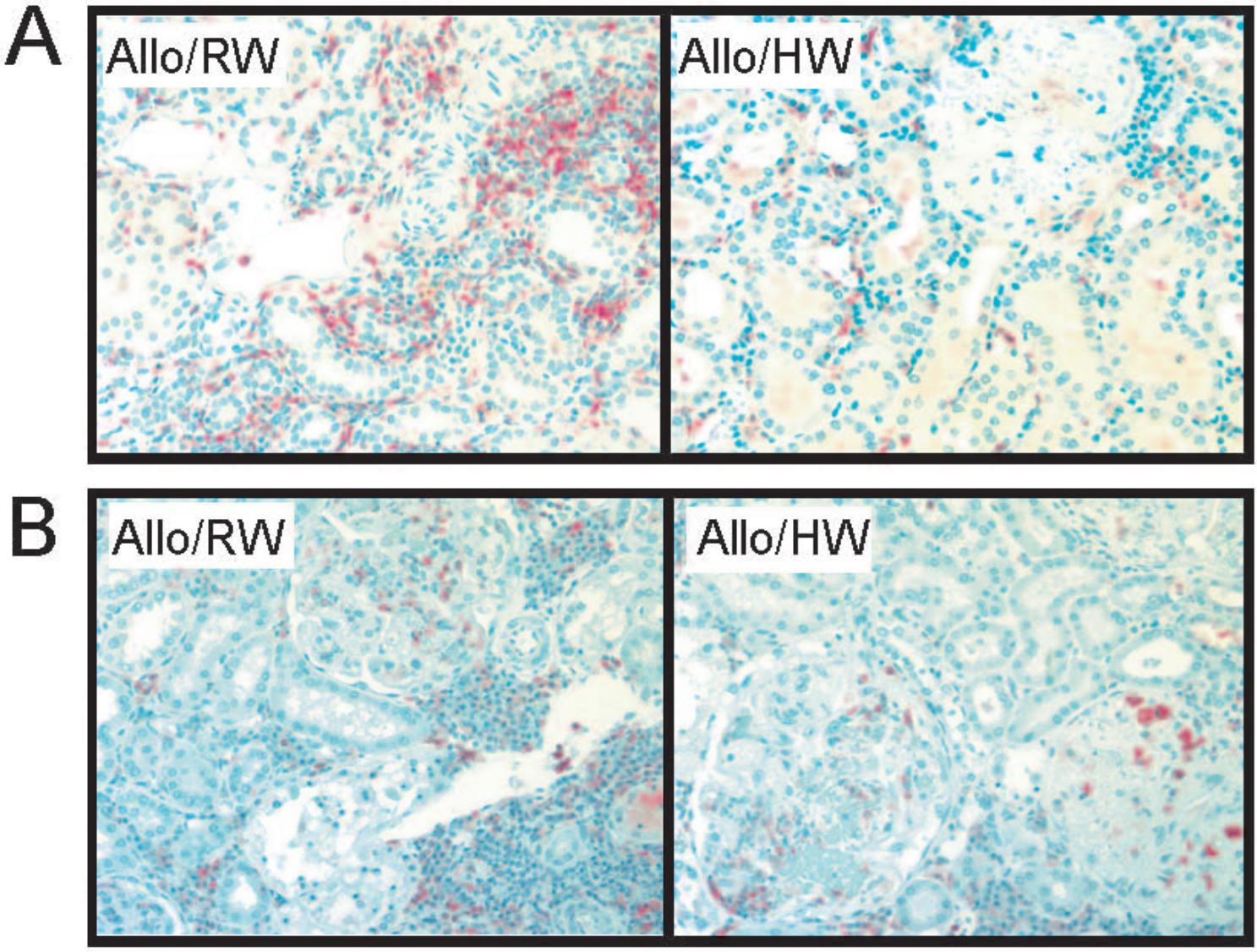

A
Allo/RW
Allo/HW
B
Allo/RW
Allo/HW

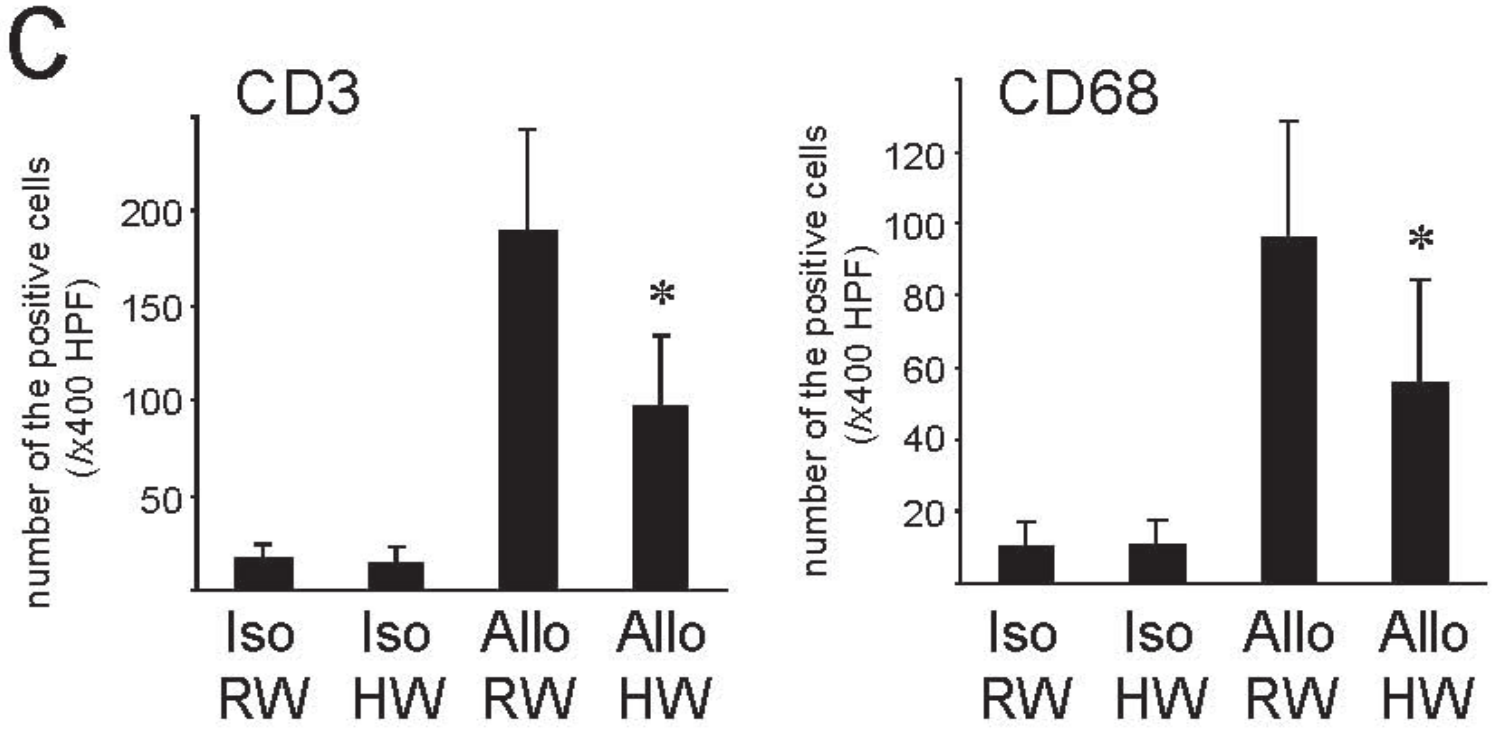

C
CD3
number of the positive cells
(/x400 HPF)
200
150
100
50
*
Iso RW
Iso HW
Allo RW
Allo HW
CD68
number of the positive cells
(/x400 HPF)
120
100
80
60
40
20
*
Iso RW
Iso HW
Allo RW
Allo HW

Figure 5

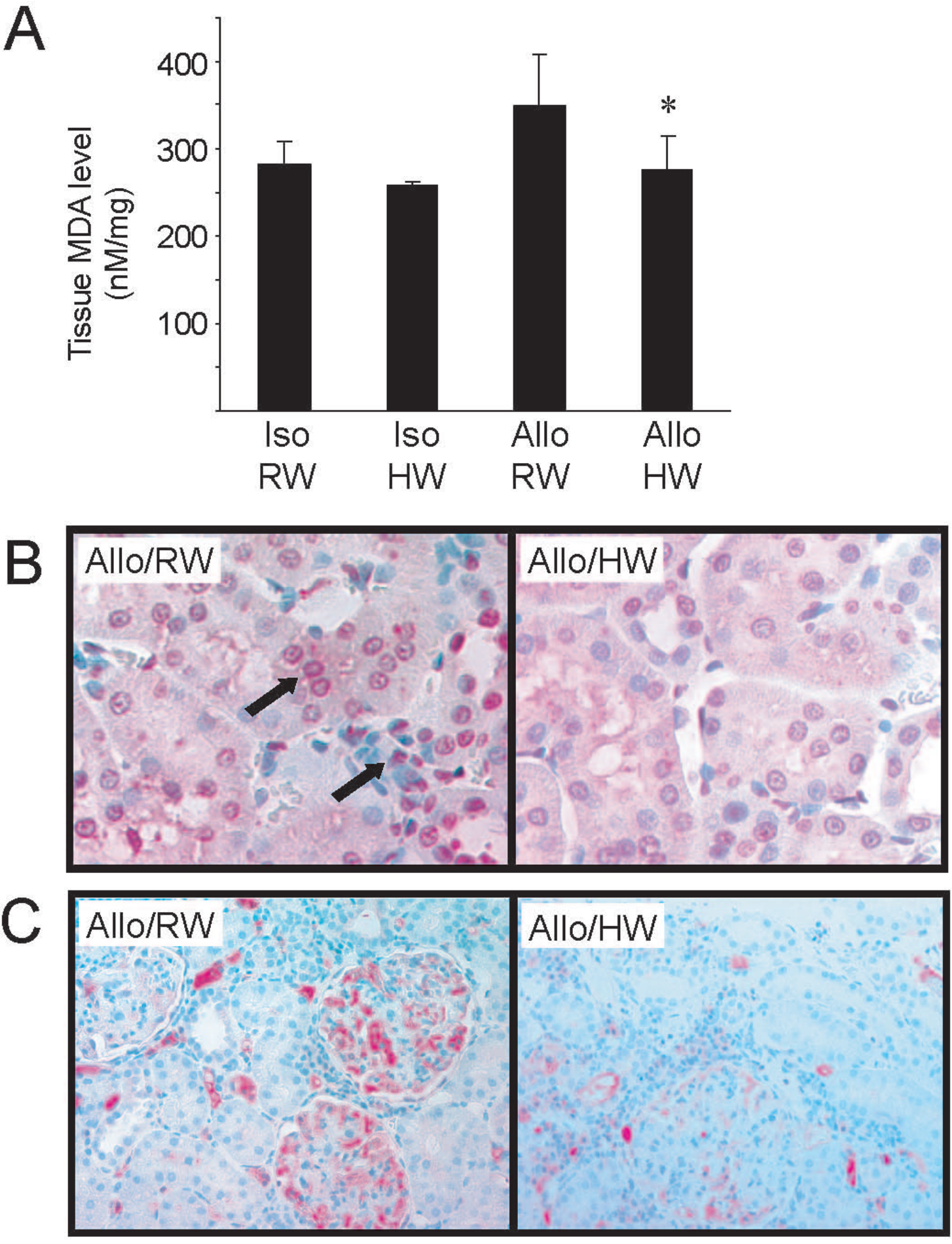

A
Tissue MDA level (nM/mg)
400
300
200
100
*
Iso RW
Iso HW
Allo RW
Allo HW
B
Allo/RW
Allo/HW
C
Allo/RW
Allo/HW

Figure 6

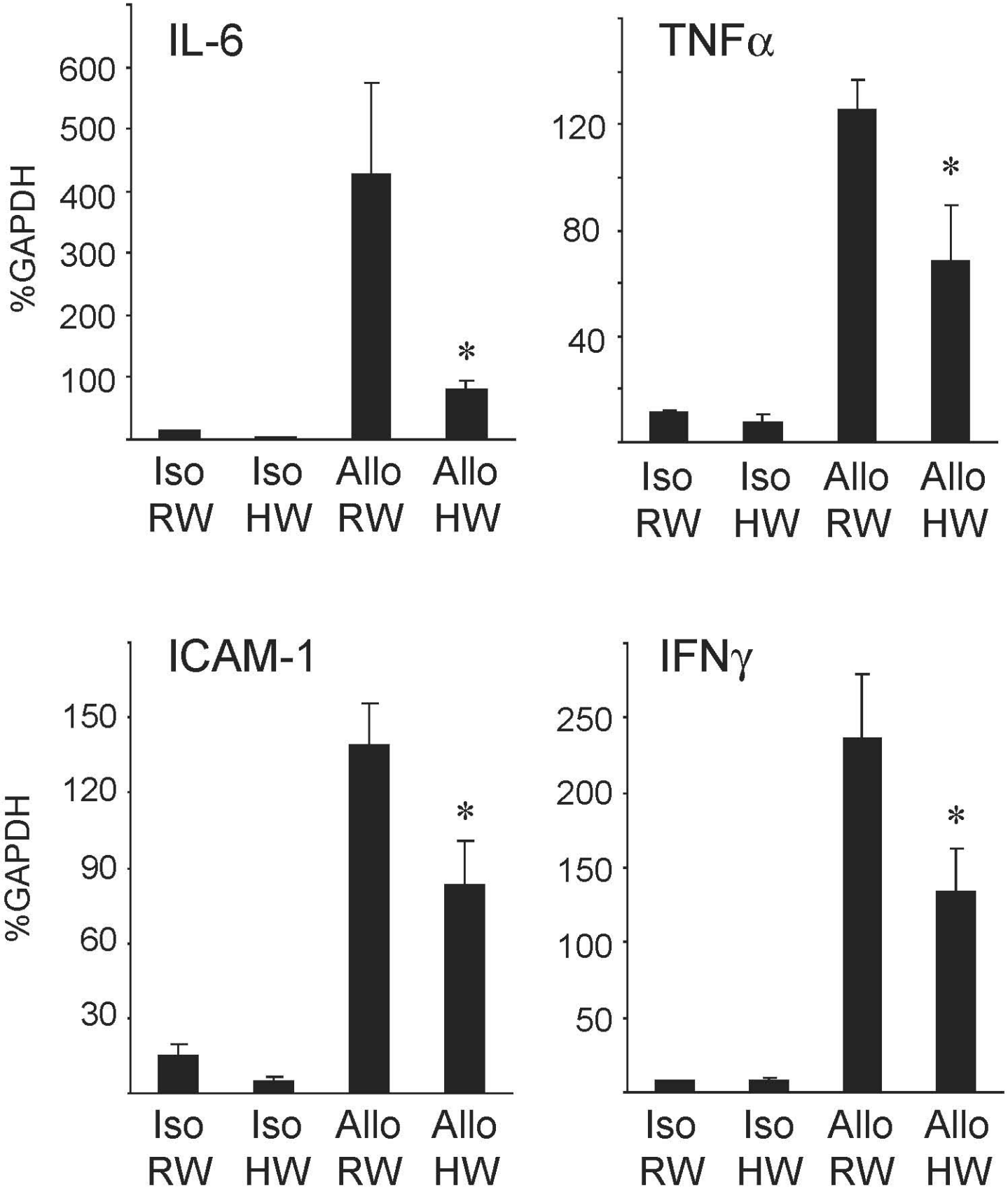
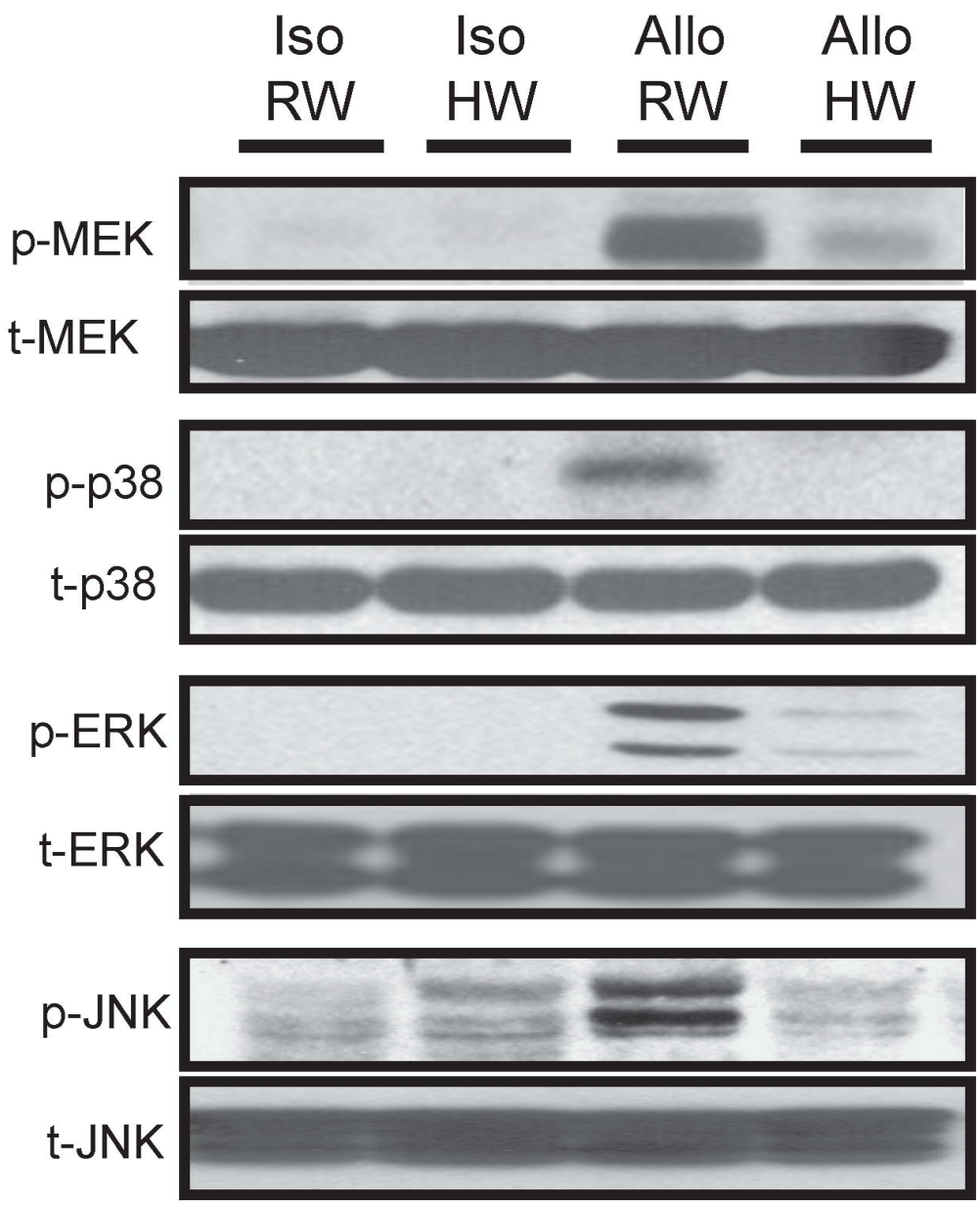

IL-6
%GAPDH
600
500
400
300
200
100
*
Iso RW
Iso HW
Allo RW
Allo HW
TNFα
120
80
40
*
Iso RW
Iso HW
Allo RW
Allo HW
ICAM-1
%GAPDH
150
120
90
60
30
*
Iso RW
Iso HW
Allo RW
Allo HW
IFNγ
250
200
150
100
50
*
Iso RW
Iso HW
Allo RW
Allo HW

Figure 7


Iso RW
Iso HW
Allo RW
Allo HW
p-MEK
t-MEK
p-p38
t-p38
p-ERK
t-ERK
p-JNK
t-JNK